Environmental Health Perspectives
Vol. 69, pp. 59-65, 1986

Carcinogenicity of By-Products of Disinfection in Mouse and Rat Liver

by Sydna L. Herren-Freund* and Michael A. Pereira*

By-products of disinfection were tested for initiating and/or promoting activity in rat liver by using the rat liver foci bioassay. The assay uses an increased incidence of γ-glutamyltranspeptidase-positive foci (GGT foci) as an indicator of carcinogenicity. The by-products of disinfection, including chloramine, halogenated humic acids, halogenated ethanes, halogenated acetonitriles, halogenated methanes, halogenated ethylenes, and N-Cl-piperidine, did not initiate GGT foci, which would indicate that they are not capable of initiating carcinogenesis. Chloroform and halogenated benzenes were tested in this assay for their ability to promote the occurrence of GGT foci and tumors initiated by diethylnitrosamine (DENA). Chloroform (1800 ppm in the drinking water) either had no effect or inhibited the occurrence of GGT foci when administered subsequent to a single dose of DENA. However, when the chloroform was administered in drinking water concurrently with weekly doses of DENA, it enhanced the formation of liver tumors. Of 20 halogenated benzenes tested, only 1,2,4,5-tetrachlorobenzene and hexachlorobenzene promoted the occurrence of DENA-initiated GGT foci. Thus in rat liver, the tested by-products of drinking water disinfection did not demonstrate tumor-initiating activity, although a few appeared to possess tumor-promoting activity.

Chloroform was also tested for tumor-promoting activity in 15-day-old Swiss mice initiated with ethylnitrosourea (ENU). At weaning they started to receive either 1800 ppm chloroform or 500 ppm sodium phenobarbital (the positive control for tumor promotion) in their drinking water. The mice continued to receive either chloroform or phenobarbital until 51 weeks of age and were sacrificed at 52 weeks of age. ENU at 5 and 20 μg/g caused a dose-dependent increase in liver tumors. In male mice, chloroform inhibited both spontaneous and ENU-induced liver tumors. When administered in the drinking water, chloroform inhibited, whereas phenobarbital promoted, hepatocarcinogenesis in mice.

Introduction

Experimental chemical carcinogenesis is generally considered to be a multistage process consisting of at least the stages of initiation and promotion. Initiation-promotion was first observed in mouse skin, where an increased yield of tumors resulted from repeated applications of croton oil to skin that had been previously exposed to a single subcarcinogenic dose of benzo(a)pyrene (*1–3*). Initiation-promotion has been described in tissues other than skin, including liver, bladder, and stomach (*4–9*). Initiation occurs when a carcinogen binds to the DNA followed by fixation of the alteration during cellular replication (*10,11*). The altered genotype can be observed as an altered phenotype. Promotion is believed to result in clonal expansion of the initiated cell (*12*). This clonal expansion can occur through direct stimulation of cellular replication of the initiated cells or through indirect stimulation of initiated cells as the result of the inhibition of cellular replication in the surrounding noninvolved cells. Clonal expansion results in a focus of cells possessing the altered phenotype.

*Health Effects Research Laboratory, U. S. Environmental Protection Agency, Cincinnati, OH 45268.

An initiation-promotion bioassay called the rat liver foci bioassay is being developed in rodent liver to detect chemical carcinogens (*13–16*). The endpoint of the assay is the occurrence of altered foci of hepatocytes that are putative preneoplastic lesions. The occurrence of these altered foci depends on the administration of a carcinogen or an initiator and can be enhanced by subsequent administration of a promoter (*15,16*). These foci are readily identified histochemically as focal areas with increased γ-glutamyltranspeptidase (GGT) activity, decreased adenosine triphosphatase activity, decreased glucose-6-phosphatase activity, or a resistance to iron accumulation in siderotic liver. The initiation-promotion bioassay using GGT-foci has detected the initiating activity of both hepatic and nonhepatic carcinogens (*17*). It has also detected the promoting activity of chemicals such as barbital (*18*), hexachlorobenzene (*19*), lindane (*19*), mestranol (*20*), phenobarbital (*18–27*), polychlorinated biphenyls (*22,23,28*) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*29*). Thus we used this assay to assess the initiating and promoting ability of drinking water disinfection by-products.

The administration of ethylnitrosourea (ENU) to 15-day-old mice has been shown to be an effective procedure for initiating liver tumors (*30,31*), and the admin-

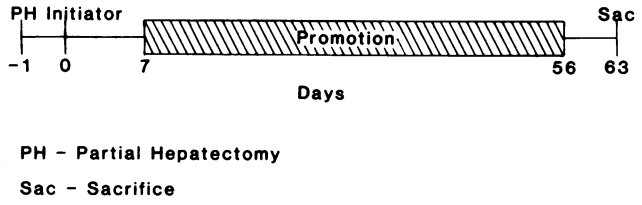

FIGURE 1. Protocol for the liver foci bioassay.

istration of phenobarbital has been shown to be an effective procedure for promoting mouse hepatocarcinogenesis (*31–34*). The administration of chloroform in corn oil by gavage induced liver tumors in B6C3F1 mice (*35*). Chloroform has been shown to lack or at most possess minimum genotoxic activity (*31,36–42*) and has also been shown to not bind significantly to liver or kidney DNA in rats (*43–45*). These findings have led to the proposal that the hepatocarcinogenicity of chloroform in mice results from a nongenotoxic mechanism such as tumor promotion (*45,46*). We therefore attempted to demonstrate the hepatic tumor-promoting activity of chloroform in mice.

Materials and Methods

Animals

Male Sprague-Dawley (CD-1) rats, male Fischer 344 rats, and male Swiss (CD-1) mice were purchased from Charles River Co. (Portage, MI). The animals were fed Purina Laboratory Rodent Chow (Ralston Purina Co., St. Louis, Mo) and given drinking water *ad libitum*. The animals were maintained according to the standards set forth in The Guide for the Care and Use of Laboratory Animals (*46*).

Chemicals

Diethylnitrosamine (DENA) was purchased from Eastman Kodak Co. (Rochester, NY); sodium phenobarbital (U.S.P.) from either Mallinckrodt Inc. (St. Louis, MO) or J. T. Baker (Phillipsburg, NJ); optimum cutting temperature compound and hematoxylin from Fisher Scientific Co. (Pittsburg, PA); *N*-γ-L-glutamyl-4-methoxy-2-naphthylamide from Bachem (Torrance, CA); ENU (purity >95%) from Sigma Chemical Co. (St. Louis, MO); and chloroform (glass-distilled, nonpreservative, and purity >99%) from Burdick and Jackson Laboratories Inc. (Muskegon, MI).

Table 1. Carcinogens that exhibit tumor-initiating activity in the rat liver foci bioassay used in our laboratory.

Carcinogen	Reference
2-Acetylaminofluorene	(*17*)
Aflatoxin B_1	(*17*)
Benzo(a)pyrene	(*17*)
7,12-Dimethylbenz(a)anthracene	(*17*)
1,2-Dimethylhydrazine	(*17*)
Ethyl carbamate (urethane)	(*17*)
Methylmethane sulfate	(*55*)
N-Methyl-N-nitro-N-nitrosoguanidine	(*17*)
N-Methyl-N-nitrosourea	(*55*)
N-Nitrosodiethylamine	(*16, 17*)
N-Nitrosodimethylamine	(*17, 55*)

Experimental Design

Rat Liver Foci Bioassay. Unless otherwise stated, these studies followed the protocol outlined in Figure 1. The rats were treated with the test compound 18 or 24 hr after a ⅔ partial hepatectomy. The positive control for initiation was DENA. Seven days after initiation, promotion by 500 ppm phenobarbital in the drinking water was begun. After 10 weeks of exposure to phenobarbital, the rats were removed from the exposure to the promoter for 1 week and then sacrificed. At sacrifice, 10 × 10 × 2 mm blocks of liver tissue were removed, frozen in optimum cutting temperature compound, cut into 8-μm sections, and stained for the presence of GGT activity according to the procedure of Rutenburg et al. (*47*). The sections were analyzed for the incidence of GGT foci and other lesions.

When the ability of a chemical to promote hepatocarcinogenesis was evaluated, DENA was used as the initiator, and phenobarbital was used as the positive control for promotion. Starting 1 week after the administration of the DENA, the test chemical was given over a period of at least 8 to 10 weeks. In one study, promotion was continued until 32 weeks, at which time tumors were observed. As with studies in which the initiating activity of drinking water disinfection byproducts were tested, the incidence of GGT foci and other lesions was determined.

Tumor Promotion Assay of Chloroform in Mouse Liver. The protocol for this experiment has been previously published (*31*). Briefly, 15-day-old mice were administered by intraperitoneal (IP) injection either 20, 5, or 0 μg ENU/g body weight. After weaning (5 weeks of age), the pups were divided according to sex and were randomly assigned to one of the following groups: group 1, 20 μg ENU/g; group 2, 5 μg ENU/g; group 3, 0 μg ENU/g; group 4, 20 μg ENU/g followed by 1800 ppm chloroform; group 5, 5 μg ENU/g followed by 1800 ppm chloroform; group 6, 0 μg ENU/g followed by 1800 ppm chloroform; group 7, 20 μg ENU/g followed by 500 ppm sodium phenobarbital; group 8, 5 μg ENU/g followed by 500 ppm sodium phenobarbital; and group 9, 0 μg ENU/g followed by 500 ppm sodium phenobarbital. The sodium phenobarbital and the chloroform were administered from weaning until the mice were 51 weeks old. The mice were sacrificed at 52 weeks of age, and a complete necropsy was conducted on each mouse.

Results and Discussion

The rat liver foci bioassay has been proposed for use in determining whether a chemical or a complex mixture possesses tumor-initiating activity (*16,48*). The assay has detected the tumor-initiating activity of 69% of the

Table 2. Initiating activity of by-products of drinking water disinfection.[a]

Chemical	Dose, mmole/kg	Time after PH, hr[b]	GGT foci/cm^2[c]	(n)[d]
Halogenated methanes				
Bromoform	1.0	24	0.26 ± 0.10[c]	(9)
Chloroform	2.2	24	0.43 ± 0.15	(9)
	1.1	24	0.59 ± 0.17	(8)
Carbon tetrachloride	6.06	24	0.55 ± 0.19	(10)
	3.03	24	0.63 ± 0.16	(9)
Halogenated ethanes				
1,2-Dibromoethanes	0.28	24	1.34 ± 0.77	(2)
	0.14	24	1.25 ± 0.25	(7)
	0.1	24	1.09 ± 0.65	(10)
	0.19	18	0.06 ± 0.04	(10)
	0.19	12	0.00 ± 0.00	(8)
1,1-Dichloroethane	7.33	12	0.33 ± 0.01	(10)
	7.33	18	0.54 ± 0.14	(10)
1,2-Dichloroethane	3.89	12	0.11 ± 0.08	(10)
	3.89	18	0.20 ± 0.07	(10)
Halogenated ethylenes				
Tetrachloroethylene	17.8	24	0.29 ± 0.13	(8)
	8.89	24	0.28 ± 0.11	(10)
Trichloroethylene	12.48	24	0.21 ± 0.09	(10)
	6.24	24	0.30 ± 0.16	(9)
cis-1,2-dichloroethylene	6.5	24	0.50 ± 0.25	(10)
	6.5	12	0.03 ± 0.03	(9)
	6.5	18	0.17 ± 0.08	(10)
	3.25	24	0.30 ± 0.09	(10)
trans-1,2-dichloroethylene	6.5	24	0.53 ± 0.15	(10)
	6.5	12	0.08 ± 0.05	(9)
	6.5	18	0.10 ± 0.10	(10)
	3.25	24	0.35 ± 0.13	(9)
Vinylidene chloride	0.69	24	0.74 ± 0.28	(10)
	0.34	24	0.91 ± 0.17	(9)
	0.70	12	0.21 ± 0.09	(9)
	0.70	18	0.12 ± 0.06	(10)
Halogenated acetonitriles				
Dibromoacetonitrile	2.0	24	1.00 ± 0.29	(6)
Dichloroacetonitrile	2.0	24	0.20 ± 0.12	(5)
Monchloroacetonitrile	1.0	24	0.46 ± 0.20	(10)
Trichloroacetonitrile	1.0	24	0.00 ± 0.00	(7)
Halogenated humic acids	2 mL/rat, pH 6.5–7.5	24	0.04 ± 0.04	(10)
Chloramine	14.75 mg/kg	24	0.23 ± 0.10	(9)
N-Cl-Piperidine	40 mg/kg	24	0.80 ± 0.32	(9)
	40 mg/kg	18	0.43 ± 0.13	(9)
	40 mg/kg	12	0.21 ± 0.11	(10)
Diethylnitrosamine	0.5	18	9.27 ± 1.31	(10)
Tricaprylin	2 mL/kg	18	0.17 ± 0.15	(10)

[a] The protocol for the rat liver bioassay used to determine tumor initiating activity is presented in Fig. 1.
[b] The time the animals are administered the test substance following a 2/3 partial hepatectomy (PH).
[c] The results are expressed as the mean ± standard error of the mean.
[d] The number of animals.

carcinogens tested, whereas none of the tested noncarcinogens were active in the assay (*49*). The only carcinogens that were not detected in the assay for tumor-initiating activity were either nongenotoxic or were direct-acting carcinogens (*7,49*). Because initiating activity in the rat liver foci bioassay depends on the genotoxic activity of the test substance, the lack of activity by nongenotoxic chemicals in the assay was not unexpected. Table 1 contains a list of the hepatic and nonhepatic carcinogens that have been tested for tumor-initiating activity in our laboratory using the protocol of the experiments described in this report. All these carcinogens exhibited tumor-inititating activity in the rat liver foci bioassay. It is recognized that the relationship between carcinogenesis and GGT foci and other altered foci used as endpoints in the assay has yet to be proved. Even so, the ability of the rat liver foci bioassay to distinguish carcinogens from noncarcinogens and to detect the initiating activity of hepatic and nonhepatic carcinogens makes it suitable for determining whether a chemical or a complex mixture possesses tumor-initiating activity.

The results from the rat liver foci bioassay for the initiating activity of drinking water disinfection by-products are presented in Table 2. Of all the chemicals found in drinking water, including disinfection by-products that were tested for initiating activity, only 1,2-dibromoethane when given 24 hr after a partial hepa-

Table 3. Induction of GGT foci following short-term exposure to chloroform or phenobarbital.[a]

DENA	Promoter	GGT foci/cm^2	(*n*)
+	$CHCl_3$	0.11 ± 0.11	(6)
+	PB	2.12 ± 0.43	(10)
+	—	0.08 ± 0.08	(8)
—	$CHCl_3$	0.43 ± 0.14	(8)
—	PB	1.38 ± 0.26	(9)
—	—	0.05 ± 0.05	(9)

[a] The protocol for the rat liver foci bioassay used is presented in Fig. 1. The animals received a ⅔ partial hepatectomy followed 1 day later by 0.3 mmole/kg DENA. Seven days later, they started to receive either 1800 ppm chloroform or 500 ppm phenobarbital in drinking water for a total of 8 weeks. The animals were sacrificed at the termination of the exposure to the promoters.

[b] The results are expressed as the mean ± standard error of the mean.

[c] The number of animals.

tectomy exhibited initiating activity. The initiating activity of 1,2-dibromoethane was minimal in comparison to the activity observed with the DENA (the positive control). Milks et al. (*50*) observed in a similar rat liver foci bioassay that 1,2-dibromoethane did not initiate GGT foci. Our *in vivo* initiation-promotion bioassay results also conflict with a previous study by Hatch et al. (*51*) in which chlorinated methanes and ethanes enhanced viral tramsformation of Syrian hamster embryo cells. Some of the chemicals tested were chloroform, 1,2-dichloroethane, and 1,1-dichloroethane. Although the *in vitro* data indicate that some of the compounds may enhance cell transformation and therefore possibly carcinogenesis, the *in vivo* data indicate that they do not initiate carcinogenesis. In conclusion, disinfection by-products, with the possible exception of 1,2-dibromoethane, did not induce GGT foci, which indicates that they do not possess significant ability to initiate carcinogenesis.

Chloroform administered in corn oil has been shown to induce hepatocellular carcinomas in mice and epithelial tumors in the kidney of male rats (*35*). The inability of chloroform to bind significantly to liver and kidney DNA (*44–46*) and its lack or very low level of genotoxicity (*31,36–43*) has led to the proposal that the carcinogenicity of chloroform results from a nongenotoxic mechanism such as tumor promotion (*45,46*). Because the mechanism of carcinogenicity (i.e., genotoxic or nongenotoxic) of a substance has major implications in interspecies and low-dose extrapolation, we attempted to demonstrate the nongenotoxic and tumor-promoting mechanism for the carcinogenic activity of chloroform.

The ability of chloroform ($CHCl_3$) to promote the appearance of GGT foci was evaluated in male Fischer 344 rats (Table 3). The rats received a ⅔ partial hepatectomy 1 day before being treated with DENA (0.3 mmole/kg body weight [bw]); 7 days later these rats were started on chloroform (1800 ppm) in drinking water. Phenobarbital (PB) (500 ppm) in drinking water was used as a positive control. Exposure to the promoters was continued for 8 weeks, and the animals were then sacrificed. Phenobarbital enhanced the incidence of GGT foci, whereas chloroform did not alter the incidence of GGT foci/cm^2.

Chloroform was then tested in male Sprague-Dawley rats for its ability to act as a cocarcinogen (Table 4). Chloroform (1800 ppm in the drinking water) was given concurrently with weekly doses of DENA (8.2 mg/kg body weight). Concurrent administration of phenobarbital (500 ppm) with DENA was used as the positive control. Treatment was continued for either 16 or 32 weeks, at which time the rats were sacrificed. At neither time point did co-administration of chloroform increase the incidence of GGT foci over the incidence observed in animals that received only DENA or over the level in animals that received only the water vehicle control (Table 4). Phenobarbital did increase the incidence of GGT foci when co-administered with DENA. The concurrent administration of either chloroform or phenobarbital with DENA did not affect the incidence of animals with tumors or the number of tumors per animal. Hence, phenobarbital greatly increased the incidence of GGT foci without altering the yield of tumors induced by DENA. This finding indicates that a quantitative relationship between the incidence of GGT foci and the incidence of tumors does not exist in all experimental protocols. Thus, a substance under certain experimental situations can increase the incidence of GGT foci without altering the incidence of tumors.

The effect of administering chloroform subsequent to ethylnitrosourea (ENU) initiation in neonatal mice was investigated (*31*). Fifteen-day-old CD-1 Swiss mice received IP injections of either 20, 5, or 0 μg/g ENU dissolved in 1.0 M sodium acetate (pH 5.6). After wean-

Table 4. Foci and tumor incidence with concurrent administration of chloroform[a].

		GGT-foci/cm^2[b]		Tumors (32 wks)[b]	
DENA	Co-carcinogen	16 wk	32 wk	% Animals with tumors	No. of tumors per animal
+	$CHCl_3$	0.13 ± 0.08 (8)	2.76 ± 0.50 (12)	83 (12)	1.3 ± 0.33
—	$CHCl_3$	0.00 ± 0.00 (8)	0.00 ± 0.00 (12)	0 (12)	0.0 ± 0.0
+	Phenobarbital	3.65 ± 1.51 (6)	23.58 ± 3.10 (11)	60 (10)	1.4 ± 0.45
—	Phenobarbital	0.14 ± 0.09 (8)	0.34 ± 0.11 (12)	0 (12)	0.0 ± 0.0
+	—	0.16 ± 0.10 (8)	3.08 ± 0.58 (10)	60 (9)	0.8 ± 0.25
—	—	0.00 ± 0.00 (8)	0.03 ± 0.03 (12)	0 (12)	0.0 ± 0.0

[a] The rats received weekly doses of 8.2 mg/kg DENA concurrently with either 1800 ppm chloroform or 500 ppm sodium phenobarbital in their drinking water. The animals were sacrificed at either 16 or 32 weeks after the start of treatment (16 or 32 doses of DENA, respectively).

[b] The results are expressed as the mean ± standard error of the mean. The number of animals is in parentheses.

Table 5. Effect of subsequent treatment with chloroform or phenobarbital on the incidence of liver tumors initiated by ENU in 15-day-old male mice.

Group	ENU treatment, mg/kg	N	Body weight, g[b]	No. of animals with altered foci areas	No. of animals with tumors[a]	No. of animals with adenomas[c]	Adenomas per animal[b]	No. of animals with carcinomas[c]	Carcinomas per animal[b]
	ENU								
1	20	30	42.4 ± 1.1	23	22[c]	22	3.13 ± 0.57	10	0.83 ±0.24
2	5	39	40.4 ± 0.7	16	8	8	0.51 ± 0.20	2	0.10 ±0.08
3	0	37	45.5 ± 0.7	3	2	2	0.19 ± 0.14	2	0.08 ±0.06
	ENU + 1800 ppm chloroform								
4	20	29	38.0 ± 0.9	17	12†	12**	1.00 ± 0.31‡	5*	0.21 ± 0.14†
5	5	25	37.3 ± 1.1	7	1†	1**	0.04 ± 0.04†	0	0.00 ±0.00
6	0	23	38.0 ± 0.8*	0	0	0	0.00 ± 0.00	0	0.00 ±0.00
	ENU + 500 ppm phenobarbital								
7	20	25	45.7 ± 1.0	21	24	22	2.12 ± 0.30	17†	2.48 ±0.4
8	5	36	41.9 ± 1.7	14	17*	14	0.56 ± 0.16	10‡	0.42 ±0.1
9	0	30	43.2 ± 0.6	4	11†	6	0.27 ± 0.10	6	0.23 ±0.0

[a] Includes the number of animals with adenoma and/or carcinomas.
[b] Results are mean ± standard error.
[c] The results expressed as number of animals with either tumors, adenomas, or carcinomas were analyzed by Fisher's exact test, and the results expressed as adenomas/animal or carcinomas/animal were analyzed by Student's *t*-test. The results of the ENU + chloroform groups and the ENU + phenobarbital groups were compared to the corresponding ENU group.
*$p \leq 0.10$.
†$p \leq 0.05$.
‡$p \leq 0.01$.

ing, the pups were separated into groups and began to receive either 1800 ppm chloroform or 500 ppm phenobarbital in drinking water. Phenobarbital was used as the positive control for tumor promotion. The mice continued to receive either chloroform or phenobarbital in their drinking water until they were sacrificed at 52 weeks of age. In the liver, administration of ENU resulted in a dose-related increase in altered foci/areas, adenomas, and hepatocellular carcinomas in male mice (Table 5). Treatment with phenobarbital (500 ppm) subsequent to ENU initiation increased the total hepatocellular carcinoma incidence in male mice (Table 5).

Table 6. Promoting activity of the halogenated benzenes[a].

Chemical	Dose, mmole/kg	GGT foci/cm^2[b]	
		Male	Female
Chlorobenzene	1.0	0.67 ± 0.31 (5)	0.64 ± 0.18 (7)
o-Dichlorobenzene	1.0	0.20 ± 0.10 (8)	0.68 ± 0.35 (8)
m-Dichlorobenzene	1.0	0.00 ± 0.00 (8)	0.27 ± 0.21 (7)
1,2,3-Trichlorobenzene	1.0	0.54 ± 0.20 (7)	0.39 ± 0.39 (2)
1,3,5-Trichlorobenzene	1.0	0.61 ± 0.24 (9)	0.57 ± 0.28 (7)
1,2,4-Tetrachlorobenzene	1.0	0.09 ± 0.09 (10)	0.13 ± 0.09 (10)
1,2,3,4-Tetrachlorobenzene	0.5	0.17 ± 0.12 (8)	0.33 ± 0.26 (7)
1,2,3,5-Tetrachlorobenzene	1.0	0.00 ± 0.00 (10)	0.312 ± 0.19 (7)
1,2,4,5-Tetrachlorobenzene	0.25	1.20 ± 0.34 (10)	0.19 ± 0.19 (7)
Pentachlorobenzene	0.5	0.15 ± 0.08 (10)	ND[c]
Hexachlorobenzene	1.0	1.51 ± 0.72 (10)	1.28 ± 0.41 (10)
Bromobenzene	1.0	0.36 ± 0.18 (3)	0.14 ± 0.16 (6)
o-Dibromobenzene	1.0	0.70 ± 0.36 (7)	ND
m-Dibromobenzene	1.0	0.00 ± 0.00 (9)	ND
p-Dibromobenzene	1.0	0.00 ± 0.00 (9)	ND
1,4-Dibromobenzene	1.0	0.19 ± 0.19 (10)	ND
1,2,4-Tribromobenzene	1.0	0.10 ± 0.07 (10)	ND
1,3,5-Tribromobenzene	1.0	0.16 ± 0.10 (10)	ND
1,2,4,5-Tetrabromobenzene	1.0	0.13 ± 0.09 (9)	ND
Hexabromobenzene	0.5	0.26 ± 0.14 (9)	ND
Tricaprylin	2 ml/kg	0.17 ± 0.15 (10)	0.58 ± 0.23 (10)

[a] The rats were administered 0.5 mmole/kg DENA followed at 1 and 5 weeks with IP injection of the halogenated benzene. The animals were sacrificed 2 weeks after the last dose of the halogenated benzene was administered.
[b] The results are expressed as the mean ± standard error of the mean. The number of animals is in parentheses.
[c] Not done.

Treatment with chloroform (1800 ppm) subsequent to the ENU administration decreased the incidence of liver tumors by approximately one-half. In mice not initiated with ENU, chloroform did not induce altered foci/areas and tumors. Our results demonstrate that instead of promoting the development of liver tumors initiated by ENU administered to neonatal mice, chloroform inhibited the formation of the tumors. These results are in contrast to the NCI-NTP-sponsored bioassay in which liver tumors were found following the administration of chloroform in corn oil by stomach gavage (*35*). The observed differences between the two studies might be the result of the different strains of mice used or the result of vehicle differences (corn oil versus drinking water) (*31*). Thus the mechanism by which chloroform administered in corn oil can induce hepatocarcinogenesis in mice and by which it can inhibit hepatocarcinogenesis in mice when administered in drinking water is poorly understood. It is the conclusion of our studies that the proposed ability of chloroform to act as a tumor promoter has still not been proven.

Hexachlorobenzene has been shown to be carcinogenic in rodent liver (*52–54*). Similar to that of chloroform, its carcinogenic activity has been proposed to result from the nongenotoxic mechanism of tumor promotion. Therefore, a series of halogenated benzenes, including hexachlorobenzene, were tested for their ability to enhance the incidence of DENA-initiated GGT foci in male and female Sprague-Dawley rats (Table 6). The rats were administered DENA by gavage (0.5 mmole/kg body weight). Groups of ten rats each were then administered one of the various halogenated benzenes by IP injection at 1 and 5 weeks after the administration of DENA. Two weeks after the final dose of the halogenated benzene was administered, the rats were sacrificed. Of the various halogenated benzenes tested, only 1,2,4,5-tetrachlorobenzene and hexachlorobenzene enhanced the occurrence of GGT foci in male rats and only hexachlorobenzene enhanced the occurrence of GGT foci in female rats. These results would indicate that 1,2,4,5-tetrachlorobenzene and hexachlorobenzene can promote tumor development in rats and that the other halogenated benzenes either are not tumor promoters in rat liver, are tumor promoters by virtue of a mechanism independent of GGT foci, or are too weak to be detected by the protocol used. Validation of the tumor-promoting activity of 1,2,4,5-tetrachlorobenzene and hexachlorobenzene still requires that their enhancement of the incidence of tumors initiated by DENA be demonstrated.

This paper is being presented in honor of the retirement of Mr. Leland J. McCabe from a long and very successful government service. His guidance and support as Division Director was instrumental in the successful completion of the research described in this paper.

Some of these studies were supported in part by U.S. Environmental Protection Agency contract 68-03-3072 with the Department of Laboratory Animal Medicine at the University of Cincinnati and contract 68-03-3215 with Pathology Associates, Inc.

This paper has been subject to the Agency's review and has been approved for publication as an EPA document. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

The authors gratefully acknowledge the skillful technical assistance of Dr. Alfred Britt, Dr. Richard Long, Marlene Khoury, Marlene Schamer, Debbie Medlock, Renee Schinaman, Edith Greene, Johnnie George, Kathy Simpson, and Monica Dawkins-Smith in the performance of these studies.

REFERENCES

1. Berenblum, I. The co-carcinogenic action of croton resin. Cancer Res. 1: 44–48 (1941).
2. Berenblum, I., and Shubik, P. A new quantitive approach to the study of the stages of chemical carcinogenesis in mouse skin. Brit. J. Cancer 1: 383–391 (1947).
3. Mottram, J. C. A developing factor in blastogenesis. J. Pathol. Bacteriol. 56: 181–187 (1944).
4. Farber, E. Carcinogenesis-cellular evolution as a unifying thread. Cancer Res. 33: 2537–2550 (1973).
5. Farber, E. The pathology of experimental liver cell cancer. In: Liver Cell Cancer (H. M. Cameron, D. A. Linsell, and G. P. Warwick, Eds.), Elsevier, Amsterdam, 1976, pp. 243–278.
6. Cohen, S. M. Urinary bladder carcinogenesis: initiation-promotion. Semin. Oncol. 6: 157–160 (1979).
7. Cohen, S. M., Arai, M., Jacobs, J. B., and Freidell, G. H. Promoting effect of saccharin and *dl*-tryptophan in urinary bladder carcinogenesis. Cancer Res. 39: 1207–1217 (1979).
8. Goerttler, K., Loehrke. H., Schweizer, J., and Hesse, B. Systemic two-stage carcinogenesis in the epithelium of the forestomach of mice using 7,12-dimethylbenz(a)anthracene as initiator and the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate as promoter. Cancer Res. 39: 1293–1297 (1979).
9. Matsukura, N., Kawachi, T., Sano, T., Sasajima, K., and Sugimura, T. Promoting action of croton oil on gastrocarcinogenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in rats. J. Cancer Res. Clin. Oncol. 93: 323–327 (1979).
10. Kauffman, W. K., Kauffman, D. G., and Rice, J. M. Possible inhibition of rat hepatocyte proliferation by hydrocortisone and its effect on cell cycle-dependent hepatocarcinogenesis by *N*-methyl-*N*-nitrosourea. Cancer Res. 41: 4653–4660 (1981).
11. Columbano, A., Rajalakshmi, S., and Sarma, D. S. R. Requirement of cell proliferation for the initiation of liver carcinogenesis as assayed by three different procedures. Cancer Res. 41: 2079–2083 (1981).
12. Scherer, E. Neoplastic progression in experimental hepatocarcinogenesis. Biochim. Biophys. Acta 738: 219–236 (1984).
13. Tsuda, H., Lee, G., and Farber, E. Induction of resistant hepatocytes as a new principle for a possible short-term *in vivo* test for carcinogens. Cancer Res. 40: 1157–1164 (1980).
14. Solt, D., and Faber, E. New principle for the analysis of chemical carcinogenesis. Nature 263: 701–703 (1976).
15. Ford, J. O., and Pereira, M. A. Short-term *in vivo* initiation/promotion bioassay for chemical carcinogens. J. Environ. Pathol. Toxicol. 4: 39–46 (1980).
16. Pereira, M. A. Rat liver foci bioassay. J. Am. Coll. Toxicol. 1: 101–118 (1982).
17. Herren, S. L., Pereira, M. A., Britt, A. L., and Khoury, M. M. Initiation/promotion assay for chemical carcinogens in rat liver. Toxicol. Letters 12: 143–150 (1982).
18. Pereira, M. A., Savage, R. E., Herren, S. L., and Guion, C. W. Comparison of tumor promotion and induction of ornithine decarboxylase by barbiturates. Carcinogenesis 3: 147–150 (1982).
19. Pereira, M. A., Herren, S. L., Britt, A. L., and Khoury, M. M. Sex difference in enhancement of GGTase-positive foci by hexachlorobenzene and lindane in rat liver. Cancer Letters 15: 95–101 (1982).
20. Yager, J. D., and Yager, R. Oral contraceptives as promoters of hepatocarcinogenesis in female Sprague-Dawley rats. Cancer Res. 40: 3680–3685 (1980).
21. Ford, J. O., and Pereira, M. A. Short term *in vivo* initiation/promotion bioassay for chemical carcinogens. J. Environ. Pathol. Toxicol. 4: 39–46 (1980).

22. Nishizumi, M. Enhancement of diethylnitrosamine hepatocarcinogenesis in rats by exposure to polychlorinated biphenyls or phenobarbital. Cancer Letters 2: 11–16 (1976).
23. Nishizumi, M. Effect of phenobarbital, dichlorodiphenyltrichloroethane and polychlorinated biphenyls on diethylnitrosamine-induced hepatocarcinogenesis. Gann 70: 835–837 (1979).
24. Kitagawa, T., and Sugano, H. Enhancing effect of phenobarbital on the development of enzyme-altered islands and hepatocellular carcinomas initiated by 3'-methyl-4-(dimethylamino)azobenzene or diethylnitrosamine. Gann 69: 679–687 (1978).
25. Mochizuki, Y., Furukawa, K., Sawada, N., and Gotoh, M. Dose-dependent enhancing effect of phenobarbital on hepatocarcinogenesis initiated by diethylnitrosamine in the rat. Gann 72: 170–173 (1981).
26. Kitagawa, L., Pitot, H. C., Miller, E. C., and Miller J. A. Promotion by dietary phenobarbital of heptocarcinogenesis by 2-methyl-*N*,*N*-di-methyl-4-aminoazobenzene in the rat. Cancer Res. 39: 112–115 (1979).
27. Herren, S. L., and Pereira, M. A. Tumor promotion in rat liver. Environ. Health Perspect. 50: 123–129 (1983).
28. Preston, B. D., Van Miller, J. P., Moore, R. W., and Allen, J. R. Promoting effects of polychlorinated biphenyls (Aroclor 1254) and polychlorinated dibenzofuran-free Aroclor 1254 on diethylnitrosamine-induced tumorigenesis in the rat. J. Natl. Cancer Inst. 66: 509–515 (1981).
29. Pitot, H. C., Goldsworthy, T., Campbell, H. A., and Poland, A. Quantitative evaluation of the promotion of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin of hepatocarcinogenesis from diethylnitrosamine. Cancer Res. 40: 3616–3620 (1980).
30. Vesselinovitch, S. D., Rao, K. V. N., Mihailovich, N., Rice J. M., and Lombard, L. S. Development of a broad spectrum of tumors by ethylnitrosourea in mice and the modifying role of age, sex and strain. Cancer Res. 34: 2530–2532 (1974).
31. Pereira, M. A., Knutsen, G. L., and Herren-Freund, S. L., Effect of subsequent treatment of chloroform or phenobarbital on the incidence of liver and lung tumors initiated by ethylnitrosourea in 15 day old mice. Carcinogenesis 6: 203–207 (1985).
32. Peraino, C., Fry, R. I. M., and Stafeldt, E. Enhancement of spontaneous hepatic turmorigenesis in C3H mice by dietary phenobarbital. J. Natl. Cancer Inst. 51: 1349–1350 (1973).
33. Uchida, E., and Hirono I. Effect of phenobarbital on induction of liver and lung tumors by dimethylnitrosamine in newborn mice. Gann 70: 639–644 (1979).
34. Ward, J. M., Rice, J. M., Creasia, D., Lynch, P., and Riggs, C. Dissimilar patterns of promotion by di(2-ethylhexyl)phthalate and phenobarbital of hepatocellular neoplasia initiated by diethylnitrosamine in B6C3F1 mice. Carcinogenesis 4: 1021–1029 (1983).
35. National Cancer Institute. Carcinogenesis Bioassay of Chloroform. Natl. Tech. Inform. Service No., PB 264018/AS, Washington, DC, 1974.
36. Roe, F. J. C., Palmer, A. K., Worden, A. N., and Van Abbe, N. J. Safety evaluation of toothpaste containing chloroform. 1. Long-term studies in mice. J. Environ. Pathol. Toxicol. 2: 788–819 (1979).
37. Uehleke, H., Werner, T., Greim, H., and Kramer, M. Metabolic activation of haloalkanes and tests *in vitro* for mutagenicity. Xenobiotica 7: 393–400 (1977).
38. Simmon, V. F., Kauhanen, K., and Tardiff, R. G. Mutagenic activity of chemicals identified in drinking water. In: Progress in Genetic Toxicology (D. Scott, G. A. Bridges, and F. H. Sobels, Eds.), Elsevier/North Holland Biomedical Press, New York, 1977, pp. 249–258.
39. Report of the International Collaborative Program. Elsevier/North Holland Biomedical Press, Amsterdam.
40. Van Abbe, N. J., Green, T. J., Jones, E., Richold, M., and Roe, F. J. E. Bacterial mutagenicity studies on chloroform *in vitro*. Food Chem. Toxicol. 20: 557–561 (1982).
41. Kirkland, D. J., Smith, K. L., and Van Abbe, N. J. Failure of chloroform to induce chromosome damage or sister-chromatid exchanges in cultured human lymphocytes and failure to induce reversion in *Escherichia coli*. Food Cosmet. Toxicol. 19: 651–656 (1981).
42. Sturrock, J. Lack of mutagenic effect of halothane or chloroform on cultured cells using the azaguanine test system. Brit. J. Anaesth. 49: 207–210 (1977).
43. Diaz-Gomez, M. I., and Castro, J. A. Covalent binding of chloroform metabolites to nuclear proteins—no evidence for binding to nucleic acids. Cancer Letters 9: 213–218 (1980).
44. Pereira, M. A., Lin, L.-H. C., Lippitt, J. M., and Herren, S. L. Trihalomethanes as initiators and promoters of carcinogenesis. Environ. Health Perspect. 46: 151–156 (1982).
45. Reitz, R. H., Fox, T. R., and Quast, J. F. Mechanistic considerations for carcinogenic risk estimation: Chloroform. Environ. Health Perspect. 46: 163–168 (1982).
46. Institute of Laboraory Animal Research. Guide for the Care and Use of Laboratory Animals. National Research Council, Washington, DC.
47. Rutenburg, A. M., Kim, H., Fischbein, J. W., Hanker, J. B., Wasserkrug, H. L., and Seligman, A. M. Histochemical and ultrastructural demonstration of gamma glutamyltranspeptidase activity. J. Histochem. Cytochem. 17: 517–526 (1969).
48. Bull, R. J., and Pereira, M. A. Development of a short-term testing matrix for evaluating relative carcinogenic risk. J. Amer. Coll. Toxicol. 1: 1–16 (1982).
49. Pereira, M. A., and Stoner, G. D. Comparison of rat liver foci assay and strain A mouse lung tumor assay to detect carcinogens: a review. Fundament. Appl. Toxicol. 5: 688–699 (1985).
50. Milks, M. M., Wilt, S. R., Pereira, M. A., and Couri, D. Dibromoethane effects on the induction of gamma-glutamyltranspeptidase positive foci in rat liver. Arch. Toxicol. 51: 27–35 (1982).
51. Hatch, G. G., Mamy, P. O., Sayer, M. L., Casto, B. C., and Nenow, S. Chemical enhancement of viral transformation in Syrian hamster embryo cells by gaseous and volatile chlorinated methanes and ethanes. Cancer Res. 43: 1945–1950 (1983).
52. Cabral, J. R. P., Mollner, T., Raitano, F., and Shubik, P. Carcinogenesis of hexachlorobenzene in mice. Int. J. Cancer 23: 47–51 (1979).
53. Nagasaki, H., Tomii, S., Mega, T., Marugami, M., and Ito, N. Carcinogenicity of benzene hexachloride (BHC). In: Topics in Chemical Carcinogenesis (W. Nakahara, S. Takayama, T. Sugimura, and S. Odashima, Eds.), Univ. of Tokyo Press, Tokyo, 1972, pp. 343–353.
54. Pereira, M. A., Herren, S. L., Britt, A. L., and Khoury, M. M. Sex difference in enhancement of GGTase-positive foci by hexachlorobenzene and lindane in rat liver. Cancer Letters 15: 95–101 (1982).
55. Pereira, M. A., Herren-Freund, S. L., Britt, A. L., and Khoury, M. M. Effects of strain, sex, route of administration and partial hepatectomy on the induction by chemical carcinogens of gamma-glutamyltranspeptidase foci in rat liver. Cancer Letters 20: 207–214 (1983).